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Research Article

DEVELOPMENT AND VALIDATION OF A REVERSED PHASE-PLANAR CHROMATOGRAPHY (HPTLC) METHOD FOR DETERMINATION OF BHT AND ITS APPLICATION IN FOOD PRODUCTS

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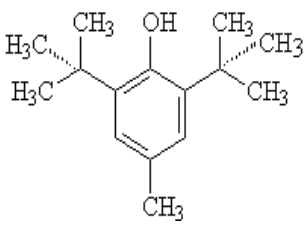
ABSTRACT

Objective: Development and validation of a RP-HPTLC method coupled with densitometry analysis for the determination of BHT as such and in commercial food sample (Butter). Methods: Chromatographic separation of BHT was achieved by employing a mobile phase consisting of Methanol – Water (9.5:0.5) on precoated aluminium TLC plates, silica gel RP-18 F254s and UV densitometric quantitative evaluation was performed by measuring the absorbance reflectance of the analyte spots at 254 nm. Results: The BHT was satisfactorily resolved with R_f value 0.48 ± 0.02. The study was carried out in the range of 500 ng to 5000 ng. The LOQ and LOD for BHT were 0.524 and 0.157 ng respectively. The method was validated with respect to linearity, repeatability, specificity, accuracy (recovery) and precision. Conclusion: The proposed method has high degree of repeatability and will provide fast and cost-effective quantitative control for routine analysis of BHT as single and in food sample.

Keywords: BHT, RP-HPTLC, Densitometry, Planar chromatography, Food product, Method Development and Validation

INTRODUCTION

Rancidification of oil and fat containing foods due to oxidation is a common problem. It affects food quality. To prevent this process antioxidants are added [1]. Butylated hydroxytoluene (BHT), chemically 2, 6-di-tert-butyl-4-methylphenol, also known as butylhydroxytoluene, is a lipophilic (fat-soluble) organic compound. It is a derivative of phenol; is useful for its antioxidant properties and used as standard for antioxidant assay [2-3]. BHT is one of the commonly used antioxidant that is found in many types of food including butter, chewing gums, baked foods and pharmaceuticals. It is used to preserve odour, colour and flavour of foods. BHT is oxidized preferentially in fats or oils and protects the foods from spoilage. There are some reports about the possible carcinogenic effect of antioxidants like BHA, BHT in high doses and long-term use [4-6]. BHT, in high doses and on long-term human use, may have potential health risks. Conversely, BHT is advocated as a dietary supplement and antiviral, useful against herpes family viruses [7]. Williams et al. 1999 [8] have reported that in lower doses BHT is not carcinogenic. The reason for BHT sufficiently non-toxic to be used in foodstuffs is that the tert-butyl groups surround the hydroxyl group. The alkyl groups being so bulky, the hydroxyl group is screened off from possibilities of chemical activity and above all from the possibility of hydrogen bonding [9]. Due to this controversial reports the detection and estimation of BHT in food products is considered important.



Chemical structure of Butylated Hydroxytoluene

Literature survey revealed that several analytical methods like HPLC [10-11], LC-MS [12- 13], GC [14-17] and GC-MS [16] have been reported for the estimation of BHT. HPTLC methods of analysis are not only reproducible, accurate and reliable but also convenient, rapid, robust and cost effective. Our literature survey did not reveal any validated HPTLC method for quantitative determination of BHT as single and as additive antioxidant in food. There is a need to develop a HPTLC method, which could be employed for the routine analysis of the BHT using simple mobile phase composition.

The present study was undertaken to develop, optimize and validate a RP-HPTLC method for analysis of BHT.

MATERIALS AND METHODS

Chemical and Solvents

Butyl hydroxyl toluene (BHT) (≥99% pure), Petroleum ether (synthesis grade), Methanol (HPLC grade) all were from Merck (India)

Chromatographic Condition

Chromatographic analysis was performed on precoated aluminium TLC plates, silica gel 60 RP-18 F254s (E. Merck, Darmstadt, Germany), using a CAMAG (Muttenz, Switzerland) Linomat 5 sample applicator. Samples were spotted in bands of 6 mm width, with a 100 µl syringe (Hamilton, Bonaduz, Switzerland). The rate of sample application was constant at 0.1 µl s^{-1}. Ascending development of the plate, with Methanol–Water (9.5:0.5) (v/v) as mobile phase, was done in a CAMAG twin-trough chamber. The plates were dried in a current of air with the help of a hair dryer. Densitometric scanning of the plate was performed with CAMAG TLC Scanner 4 at 254 nm operated by WinCATS software version 1.4.6. The source of radiation utilized was deuterium and tungsten lamp emitting a continuous UV spectrum between 200 and 700 nm. The slit dimension was 5 mm×0.45 mm and the scanning speed of 20 mm s^{-1} with 100 µm/step data resolution.

Procedure

Standard stock solutions

Accurately weighed 5 mg BHT was dissolved in 10 ml petroleum ether and the resulting stock solution (0.5 mg ml^{-1}) was used for the study.

Sample Preparation

2 g butter (procured from the local market) was extracted with 20ml petroleum ether (three times) at room temperature and the combined extract was concentrated to 5.0 ml. This solution was used for further study.

A laboratory sample was prepared by mixing 1ml of BHT stock solution with 1 ml of stock solution of butter sample (0.25 µg BHT /µl) and the same was used for accuracy study.

RESULTS AND DISCUSSION

Method Development and Optimization

Numbers of experimental parameters, such as mobile phase composition, scan modes and detection wavelengths were optimized during method development in order to obtain accurate, precise and reproducible results for the determination of BHT. Various blends of solvent systems in varying proportions were tried as mobile phase and Methanol–Water (9.5:0.5 v/v) showed better resolution of BHT (R_f 0.49) (Fig. 1). The selection of wavelength was based on maximum absorbance for optimum sensitivity.

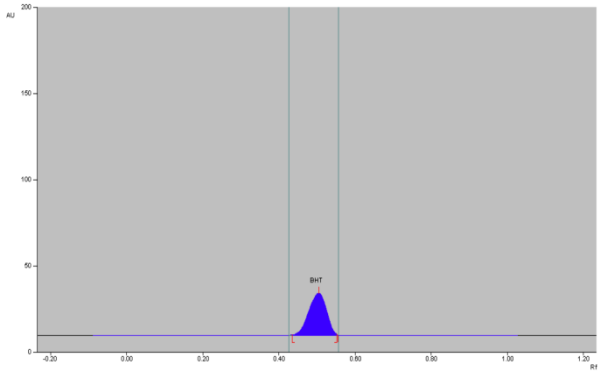


Fig. 1: Densitogram of BHT (R_f 0.49), detection at 254 nm

Analytical Method Validation

After satisfactory development of method, it was subjected to method validation to demonstrate its suitability for intended purpose. Analytical method validation was carried out by means of system suitability, accuracy, precision, linearity and solution stability.

Linearity (Calibration)

Amounts of standard solution equivalent to 2–8 µl BHT per band were applied to the prewashed HPTLC plates in order to obtain final concentrations of BHT 1000, 2000, 3000, 4000 ng spot with the help of microliter syringe and the plates were developed in twin through chamber using 10 ml freshly prepared mobile phase. After development, the plates were dried and scanned densitometrically at 254 nm as described above. Calibration plots were constructed by plotting peak area and height against the corresponding amounts of the standard (ng per band). The BHT showed good linearity in the range of 1000-4000 ng per spot. The correlation coefficients, r, were 0.9950 via area and 0.9986 via height. Calibration parameters are given in Table 1.

Table 1: Calibration data for BHT, n=4

Parameter	Result via area	Result via height
Linearity range	1000- 4000 ng	1000- 4000 ng
Correlation co-efficient	r=0.9950	r=0.9986
sdv %	3.11 %	5.93 %
Slope	113.1	5.922
Intercept	21.3	1.225

n is the number of replicates

Sensitivity

The sensitivity of measurement of BHT was estimated in terms of the limit of quantification (LOQ). The smallest amounts detected under the chromatographic conditions used were estimated in terms of the limit of detection (LOD). LOQ and LOD were calculated by using the equations LOD = 3 × N/B and LOQ = 10 × N/B, where N is the standard deviation of the peak areas of the BHT, taken as a measure of noise, and B is the slope of the corresponding calibration plot. LOQ and LOD for BHT were 0.524 and 0.157 ng, respectively.

Precision

The precision is expressed as standard deviation or relative standard deviation at intraday and inter day (n=3) analysis. Analysis was performed with three different concentration of BHT as per testing procedure. % RSD was calculated and is shown in Table 2.

Table 2: Method precision data for BHT, (n=3)

Standard	Conc. (ng)	Intra-day precision (% RSD, n=3)	Inter-day precision (% RSD, n=3)
BHT	2000	0.48 %	0.62 %
	3000	0.33 %	0.49 %
	4000	1.28 %	1.13 %

n is the number of replicates

Repeatability

The repeatability of sample application was assessed by application of 2 µl standard solutions three times to an HPTLC plate, development of plate, scanning and recording peak height and peak area for the zones. RSD (%) of peak height, peak area and R_f value were 2.497 %, 1.61 % and 1.24 % respectively for BHT (Table 3).

Table 3: Repeatability data for BHT, (n=3)

	R_f	Peak area	Peak height
Mean	0.483	119.339	6.175
SD	0.006%	1.92 %	0.154%
% RSD	1.24 %	1.61 %	2.497%

Accuracy (Recovery study)

In the initial study, no spot for BHT was detected in the butter sample. Therefore, a laboratory sample was prepared by externally adding known amount of BHT in the petroleum ether extract of sample (2.3.2) and used for the study. The accuracy of the method was determined by analysis of standard additions at three different levels, i.e. multiple-level recovery studies. Sample stock solution, which contains 2 µg BHT, was spiked with amounts equivalent to 50, 100, and 150% of amounts of sample in the original solution. (Table 4) Percentage Recovery was found to be between 99.6 – 102.7.

Table 4: Recovery study data for BHT, (n=3)

Sample	Recovery level (%)	Initial level (ng/spot)	Amount added (ng/spot)	Amount found (ng/spot)	% Recovery	% RSD
BHT	50	2000	1000	3081	102.7±0.043	1.39%
	100	2000	2000	3991	99.76±0.037	0.92%
	150	2000	3000	4980	99.60±0.044	0.88%

Specificity

To confirm the specificity of the proposed method, the solution of the sample was spotted on the TLC plate, developed and scanned. The mobile phase used resolved the samples very efficiently without any interference. (Fig.1) The R_f value of analyte was 0.49.

Typical absorption spectra of BHT are shown in Fig. 2. Peak purity for the drug was tested by acquiring spectra at the peak start (S), peak apex (M) and peak end (E) positions. Results from correlation of the three replicates spectra of BHT are r(S, M) = 0.9999 and r (M, E) = 0.9998. Therefore, it can be concluded that no impurities were eluting with the peaks obtained from the standard solution.

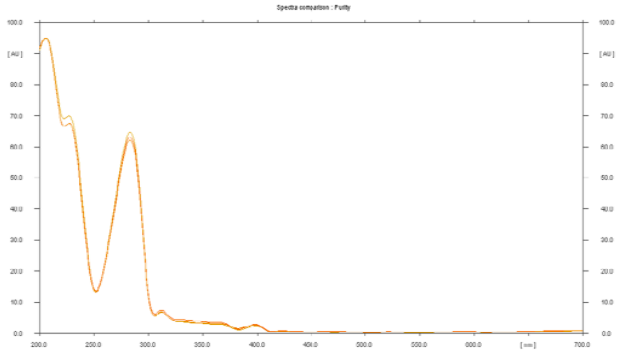


Fig. 2: Typical absorption spectra of BHT

Solutions Stability

The stability of BHT solution was investigated by leaving standard test solutions in tightly capped vial at room temperature up to 48 h. The same standard solutions were analysed initially (freshly prepared solution), after 24 h and after 48 h. The RSD (%) of the BHT peak area was calculated for the study period during solution stability experiments. The %RSD value of the BHT peak area was less than 2.0%. No significant change was observed during solution stability experiments. The results from these experiments confirm that the BHT solution used during assay was stable up to the study period of 48 h.

CONCLUSION

Use of RP-HPTLC enables analysis of samples, which are not separated in normal phase chromatography. A RP-HPTLC method for analysis of BHT has been developed. The newly developed method was found to be specific, precise, reproducible and accurate, as depicted by the statistical data of analysis for BHT. Since in this method number of samples can be analyzed simultaneously - it is less time consuming and cost effective as compare to GC and HPLC methods. It will be very useful as routine quality control method for detection and quantitative analysis of BHT as such and in different food products and pharmaceuticals.

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